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Gene expression of serotonin and dopamine receptors and monoamine oxidase-A in the brain of dominant and subordinate pubertal domestic pigs (*Sus scrofa*) fed a β-adrenoreceptor agonist☆

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ABSTRACT

Aggression is a major source of social stress with negative effects on health and well-being, yet limited information is known about the molecular mechanisms mediating aggressive behavior in swine. Ractopamine (RAC) is a β-adrenoreceptor agonist that enhances growth but increases aggressive behaviors in female pigs. Thus, the effects of RAC, sex, and social rank on the mRNA abundance of genes encoding serotonin and dopamine receptors, and monoamine oxidase (MAO)-A in brains of sub-adult pigs were evaluated. Top dominant and bottom subordinate pigs (16/sex) in pens of 4 pigs were determined, and fed either the control or RAC diets. At day 31, their raphe nuclei (RN), amygdala (AMY), frontal cortex (FC), and hypothalamus (HYP) were dissected; relative mRNA abundance for 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and D_1 receptors, and MAO-A was determined by Q-RT-PCR and data subjected to multivariate linear mixed model analysis and Tukey post-hoc test. Expression of 5-HT_{1B} and MAO-A was suppressed in the AMY of female pigs; 5-HT_{2B} expression was also suppressed in the RN, FC and HYP of females and RN of dominant pigs ($P<0.05$). Expression of 5-HT_{2A} was more up-regulated in RN of females compared to males ($P<0.05$). Expression of D_1 varied in RN and FC mostly as a function of RAC feeding and its interaction with sex and social rank ($P<0.05$). While RAC feeding is related to changes in expression of the D1 receptor mRNA, suppression in expression of serotonergic genes detected in the brain of pigs, especially in females independent of social rank, may be mediating the inter-individual offensive aggression.

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Abbreviations: AMY, amygdala; FC, frontal cortex; HYP, hypothalamus; MOA-A, monoamine oxidase A; RAC, ractopamine; RN, raphe nucleus

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1. Introduction

Elevated aggression carries the risk of injuries and even death, in addition to causing social stress and negatively affecting the health and well-being of those involved in the interaction. When access to plentiful resources is available, a stable social structure is maintained with minor episodes of aggression (Jensen, 1982; D'Eath and Turner, 2009). However, when in captivity, pigs are mostly kept in non-related groups with limited resources and, at times, mixed with unfamiliar animals (Andersen et al., 2004; D'Eath and Turner, 2009); thus, aggression associated with the formation and maintenance of social hierarchy and competition is of concern. Knowledge of the underlying mechanisms controlling aggression in pigs is limited (D'Eath et al., 2005), and any factor that may alter aggressive behavior is therefore worthy of investigation. Neurophysiological profiling of pigs can also provide a useful model for understanding aggression in higher mammal species, including humans.

Underlying mechanisms mediating aggression have been widely explored in many species including humans (Davidson et al., 2000), rodents (de Almeida et al., 2005a,b; Miczek et al., 2002), lizards (Korzan et al., 2001), birds (Buchanan et al., 1994; Dennis et al., 2008) and pigs (D'Eath et al., 2005). Dysregulation of serotonergic and dopaminergic systems in neural pathways controlling aggression trigger and follow aggressive and defensive behaviors (Miczek et al., 1994, 2002). In general, low serotonergic activity and elevated dopaminergic activity (Nikulina and Kapralova, 1992; van Erp and Miczek, 2000; de Almeida et al., 2005b; Haller et al., 2005; Nelson and Trainor, 2007) in brain areas such as the frontal cortex (FC), which provides inhibitory signaling to the amygdala (AMY) and hypothalamus (HYP), lead to aggression (Davidson et al., 2000; Nelson and Chiavegatto, 2001; Nelson and Trainor, 2007). The midbrain raphe nuclei (RN) are the primary source of the brain's serotonin and also part of this circuitry, with their serotonergic neurons projecting to the frontal and limbic regions of the brain (Carpenter, 1991). This interlinking of information across brain areas stresses the complexity of neurotransmission signaling, and thus it is worth noting that fluctuations in serotonergic and dopaminergic activity may not necessarily lead to augmented aggressive behavior (Nelson and Trainor, 2007). The behavioral outcome is also dependent upon the interaction of neurotransmitters with other molecules (e.g. steroid hormones, vasopressin, and nitric oxide), and receptor subtypes and their loci in the neurons that are in fact mediating the neurochemical signaling, along with a cascade of different signal transduction molecules via second messenger systems (Nelson and Chiavegatto, 2001).

Centrally, neurotransmitters such as serotonin and dopamine modulate aggressiveness by binding to receptors located in target-neurons across the FC, AMY, HYP, and RN among other regions (Miczek et al., 1994; Davidson et al., 2000; Boothman et al., 2003; Nelson and Trainor, 2007). Pharmacological and genetic modulations of receptor functioning such as those of 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2B} receptors predictably affect aggression (Youssef, 2004; de Boer and Koolhaas, 2005; Doly et al., 2008). Dopaminergic signaling through the dopamine receptors (D_1 and D_2) also escalates aggressive behaviors in rodents (Miczek et al., 2002; de Almeida et al., 2005b; Bondar and Kudryavtseva, 2005). The monoamine oxidase (MAO) enzymatic system deaminates synaptic serotonin and dopamine (Wells and Bjorksten, 1989; Shih et al., 1999). This enzyme may influence aggression by regulating the availability of these neurotransmitters in the brain (Shih et al., 1999; Kudriavtseva et al., 2004). Adult mice carrying a deletion in the gene encoding the MAO type A are more aggressive (Cases et al., 1995) and low MAO-A activity in cortical and subcortical brain regions correlate with the higher self-reported aggression in humans (Alia-Klein et al., 2008).

Therefore, our research focused on examining some of the underlying molecular mechanisms that has the potential to mediate aggression in pigs. We have previously showed that female pigs when supplemented with a high-tryptophan diet for 6 days reduced aggressiveness when tested with a resident–intruder test (Poletto et al., 2010c). Other studies by our group that were carried out with the same group of animals from which samples were extracted for the present study demonstrated that, compared to male pigs, female pigs displayed more offensive behaviors (bites and pursuits) during agonistic interactions while engaging more often in fights, and these behaviors were enhanced when fed the β-adrenoreceptor agonist ractopamine or RAC (Poletto et al., 2010a,b); these females also attacked with a high frequency during the resident–intruder test (Poletto et al., 2010b). Thus, we proposed that when compared to males, female pigs may have a deficiency in serotonergic signaling at the gene expression level, and social rank may be involved with these changes. In addition, noradrenergic stimulation enhances arousal and can lead to aggressive behaviors (Haller et al., 1998; Berridge, 2008), and RAC agonism has been shown to mediate similar responses in pigs (Marchant-Forde et al., 2003; Poletto et al., 2010a,b). Long-term administration of β-adrenoreceptor agonists may enhance aggression through alterations in catecholaminergic balance and neurochemical signaling mediated by D1 receptor, also a catecholaminergic G-coupled-protein receptor type. Thus, we also hypothesized that behavioral changes associated with RAC feeding (Poletto et al., 2010a,b) would relate with changes in expression of the D_1 receptor gene in the brain of pigs. Our aim was to investigate the effects of RAC feeding and sex, but also taking into account social rank (dominant and subordinate) on the mRNA abundance for the 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and dopamine D_1 receptors, in addition to MAO-A, in the AMY, FC, HYP and RN of pigs using Q-RT-PCR.

2. Results

The summary of all relevant relative changes in the expression of the genes encoding proteins for 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, D_1 receptors and MAO-A in the RN, AMY, FC and HYP respective to the effects of RAC feeding, sex, and social rank are presented in Table 1.

2.1. *Raphe nuclei gene expression profile*

Females had a 1.86 fold increase in the 5-HT_{2A} gene expression in the RN while males had an up-regulation of this gene by

Table 1 – Summary of changes in relative gene expression of the serotonin and dopamine receptors and the monoamine oxidase enzyme in the amygdala, frontal cortex, hypothalamus and raphe nuclei of pigs.

Brain area	Gene *	Effect	Relative change in expression	P-value
RN	5-HT_{2A}	Sex	↑ male vs. ↑↑female	0.001
	5-HT_{2B}	Sex	↓↓ male vs. ↓ female	0.003
	5-HT_{2B}	Social rank	↓↓ dominant vs. ↓ subordinate	0.003
	D_1	Treatment × sex	↑ CTL male vs. ↑↑ CTL and ↑↑ RAC female	<0.05
	MAO-A	Sex	↓↓ male vs. ↓ female	0.001
AMY	5-HT_{1B}	Sex	↑ male vs. ↓ female	0.04
	MAO-A	Sex	↑ male vs. ↓ female	0.06
FC	5-HT_{2B}	Sex	↓↓ male vs. ↓ female	0.02
	D_1	Treatment × social rank	↓↓ dominant CTL vs. ↓ dominant RAC	0.04
HYP	5-HT_{2B}	Sex × social rank	↑ male subordinate vs. ↓ female subordinate	0.02
	MAO-A	Sex × social rank	↓↓ female dominant vs. ↓ female subordinate and ↑ male subordinate	<0.05

Note. RN = raphe nuclei; AMY = amygdala; FC = frontal cortex; HYP = hypothalamus. Arrows pointing upwards represent up-regulation, while arrows pointing downwards represent down-regulation of the respective gene within the specified brain area. The number of arrows is proportional to the level of up- or down-regulation within the respective effect.

* 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} are classified as serotonergic receptors; D_1 is classified as a dopaminergic receptor type 1; MAO-A is defined as a monoamine oxidase type A.

1.38 fold ($P<0.01$). There was a trend for CTL-fed females to over-express the 5-HT_{2A} gene by 2.07 fold compared to CTL and RAC-fed males, which only had 1.44 and 1.31 fold increases, respectively (dietary treatment × sex, $P=0.08$); meanwhile, RAC-fed females had an increase in of 1.66 fold for 5-HT_{2A} mRNA abundance, which was not different from the other subgroups ($P>0.10$). There was a –1.65 fold decrease in the mRNA abundance for 5-HT_{2B} receptor in the RN of males compared to females, which had a slight down-regulation of –1.12 fold ($P<0.01$). Furthermore, dominant pigs had a more prominent down-regulation of 5-HT_{2B} receptor mRNA in the RN than subordinate pigs (–1.65 vs. –1.13 fold, respectively, $P<0.01$).

Female pigs that were fed both the CTL and the RAC-added diets had greater up-regulation of the dopamine D_1 expression compared to males, especially the ones receiving the CTL treatment (dietary treatment × sex, $P<0.05$; Fig. 2). Both females and males had a down-regulation of the MAO-A mRNA expression in the RN; however, this suppression was more evident in males than in females (–2.00 vs. –1.02 fold, respectively; $P<0.01$). Dominant RAC-fed pigs had the greatest up-regulation in the 5-HT_{1B} expression (2.04 fold), while subordinate RAC-fed pigs had the smallest up-regulation (1.08 fold), but there was no evidence for significant changes in expression for this pair-wise comparison ($P>0.10$; dietary treatment × social rank, $P=0.07$).

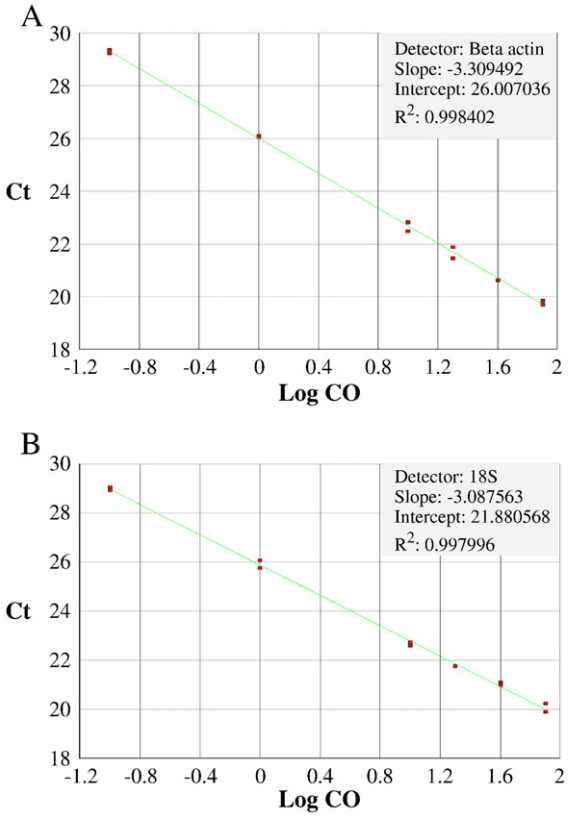

Fig. 1 – Standard curves of serial dilutions from template cDNA samples calculated by 7000 System SDS software (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). (A) Template cDNA with *Sus scrofa* beta actin gene, $R^2=0.998402$. (B) Template cDNA with *Sus scrofa* 18S rRNA gene, $R^2=0.997996$.

2.2. *Amygdala gene expression profile*

The changes in gene expression detected in the AMY were related to the mRNA abundance encoding for the 5-HT_{1B}

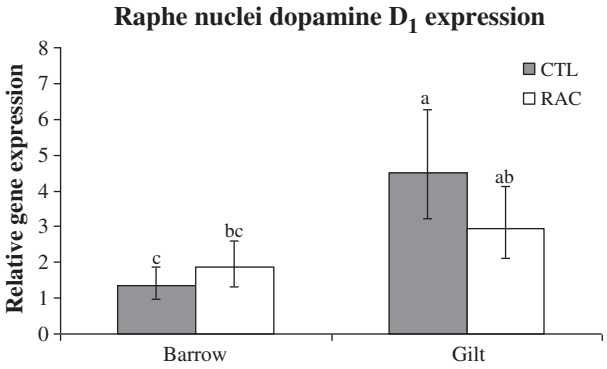

Fig. 2 – Relative expression of the dopamine D_1 receptor gene in the raphe nuclei of male and female pigs fed either the control (CTL) or the ractopamine (RAC) added diet for a period of 31 days. Positive fold-changes indicate increased mRNA levels for the respective gene in the tested brain area. The error bars represent the standard errors of the means (SEM). Dietary treatment × sex, $^{a,b,c}P<0.05$.

receptor and the enzyme MAO-A. A slight increase of 1.06 fold was found in the males' mRNA expression for the 5-HT_{1B} receptor, while females had a down-regulation of –1.34 fold on its expression ($P<0.05$). Similarly, females showed a trend towards significance for lower MAO-A mRNA expression in the AMY of –1.38 fold, while males had over-expression of 1.06 fold ($P=0.06$). There was no effect of dietary treatment, sex, social rank or on the expression of 5-HT_{2A}, 5-HT_{2B}, and dopamine D_1 ($P>0.10$).

2.3. *Frontal cortex gene expression profile*

The mRNA abundance for the 5-HT_{2B} receptor was more down-regulated in the FC of males compared to females (–2.66 vs. –1.72 fold; $P<0.05$). Dominant pigs fed the CTL diet had remarkably lower dopamine D_1 gene expression compared to dominant pigs fed the RAC-added diet, which had its expression less suppressed (dietary treatment × social rank, $P<0.05$; Fig. 3); meanwhile, no other pair-wise comparison differed ($P<0.05$; Fig. 3). Furthermore, CTL fed-females showed a tendency towards significance for lower dopamine D_1 receptor mRNA expression of –13.72 fold compared to RAC-fed females, with a –5.62 fold decrease (dietary treatment × social rank, $P=0.06$). The three-way interaction of dietary treatment, sex, and social rank was not significant ($P>0.10$). There was also no effect of dietary feeding, sex or social rank on the expression of 5-HT_{1B}, 5-HT_{2A}, and MAO-A genes ($P>0.10$).

2.4. *Hypothalamus gene expression profile*

Subordinate males had an up-regulation while subordinate females had the greatest down-regulation of the 5-HT_{2B} gene expression in the HYP ($P<0.05$); dominant males and dominant females had a down-regulation of 5-HT_{2B} that did not differ ($P>0.10$; Fig. 4; sex × social rank, $P<0.05$). Subordinate females had the greatest down-regulation of the MAO-A gene expression and differed from dominant females ($P<0.05$), which also had a down-regulation, and subordinate males ($P<0.05$), which instead had an up-regulation in MAO-A expression (sex × social rank, $P<0.05$; Fig. 5).

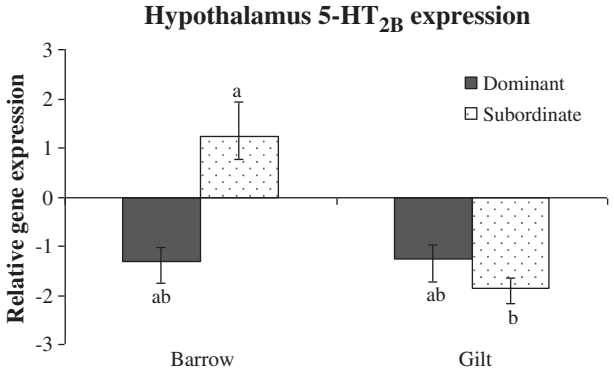

Fig. 4 – Relative expression of the 5-HT_{2B} receptor gene in the hypothalamus of dominant and subordinate male and female pigs at 6 months of age. Positive fold-changes indicate increased mRNA levels, while negative fold-changes indicate decreased mRNA levels for the respective gene in the tested brain area. The error bars represent the standard errors of the means (SEM). Sex × social rank, $^{a,b}P<0.05$.

3. Discussion

The brain areas investigated in the current study are part of the neurocircuitry responsible for regulation of impulsive and aggressive behaviors, which are mostly mediated by the modulation of the serotonergic neurotransmission and dopaminergic system (Miczek et al., 1994, 2002; Nelson and Trainor, 2007). When examining the mRNA abundance for the genes encoding the proteins for 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, dopamine D_1 receptors, and MAO-A, our results illustrate distinct regulation of their expression in the RN, AMY, FC and HYP especially in relation to sex of the pigs. Females had a suppression of gene

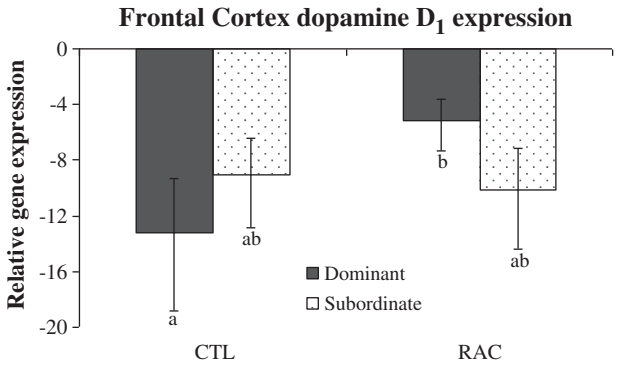

Fig. 3 – Relative expression of the dopamine D_1 receptor gene in the frontal cortex of dominant and subordinate pigs fed either the control (CTL) or the ractopamine (RAC) added diet for a period of 31 days. Negative fold-changes indicate decreased mRNA levels for the respective gene in the tested brain area. The error bars represent the standard errors of the means (SEM). Dietary treatment × social rank, $^{a,b}P<0.05$.

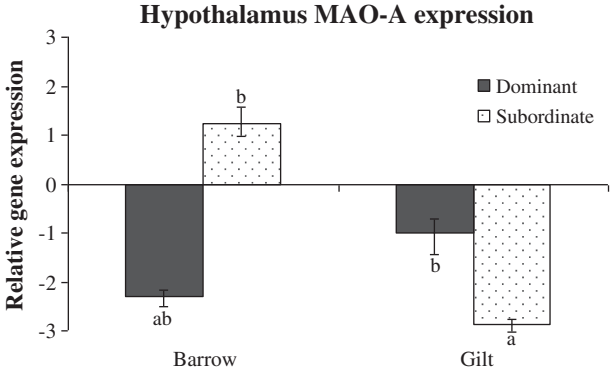

Fig. 5 – Relative expression of the enzyme MAO-A gene in the hypothalamus of dominant and subordinate males and females at 6 months of age. Positive fold-changes indicate increased mRNA levels, while negative fold-changes indicate decreased mRNA levels for the respective gene in the tested brain area. The error bars represent the standard errors of the means (SEM). Sex × social rank, $^{a,b}P<0.05$.

expression for most of the serotonergic receptors and the MAO-A in several brain areas; while relative expression of the dopamine receptor changed according to RAC feeding and its interaction with social rank and sex in the FC and RN respectively. Among the four brain areas examined, the RN had changes in the expression of all genes tested, especially in relation to sex of the pigs. To our knowledge, the relative changes in mRNA abundance for 5-HT_{2B}, dopamine D_1 receptors, and the MAO-A in the brain areas tested in our study has not been previously reported in pigs. Because brain samples were collected at one point in time, conclusions cannot be drawn regarding whether these changes in receptor expression are permanent, and whether they have been affected by uncontrolled variables such as puberty. The brain samples examined in the present study belonged to pigs that were previously tested for aggressiveness using the resident–intruder test and subjected to aggression evaluation in the home pen (Poletto et al., 2010a,b). We have demonstrated these female pigs, regardless of social rank, were more aggressive than males, and that feeding the RAC-added diet further enhanced aggressive behavior in female, but not in male pigs (Poletto et al., 2010a,b).

Under intensive farming and lab conditions, pigs are mostly kept in non-related groups that are temporarily unstable as they are mixed with unfamiliar animals (Andersen et al., 2004; D'Eath and Turner, 2009). This leads to frequent aggressive events that are associated with formation and maintenance of social hierarchy, and competition for resources (e.g. feeder access) that are generally limited (Andersen et al., 2004; D'Eath and Turner, 2009). According to Kudriavtseva et al. (2004), repeated experience of aggression results in the activation of the dopaminergic system, but inhibition of the serotonergic system in mice. Several studies carried out on humans, primates, rodents, dogs and other mammals have demonstrated a solid relationship of 5-HT_{1B} with aggression (i.e. Saudou et al., 1994; de Almeida et al., 2005a,b; de Boer and Koolhaas, 2005; Sari, 2004). The receptors 5-HT_{2A} (i.e. Olivier et al., 1995; Peremans et al., 2003), the dopamine receptor D_1 (Miczek et al., 2002; Bondar and Kudryavtseva, 2005; de Almeida et al., 2005b) and the enzyme MAO-A (Cases et al., 1995; Alia-Klein et al., 2008) have also been implicated in the control of aggression.

The function of the 5-HT_{1B} receptor in aggression regulation is well known. The strongest evidence of its role is a transgenic study in mice lacking functional expression of the gene encoding for the 5-HT_{1B} receptor; these mice showed more aggressive behavior than the wild-type (Saudou et al., 1994). Centrally, the 5-HT_{1B} receptor is expressed at the axon terminal acting as an autoreceptor, regulating the serotonergic tone in many brain regions, including those involved in the aggression neurocircuitry (Sari, 2004; Nelson and Trainor, 2007). But more importantly, pharmacological studies with rats on 5-HT_{1B} receptor action postsynaptically have strongly supported the serotonin deficiency hypothesis of aggressive behavior (de Boer and Koolhaas, 2005). The porcine sequence for the 5-HT_{1B} receptor is highly homologous (88–95%) to other animal species, with 95% homology to the human 5-HT_{1B} receptor (Bhalla et al., 2001). The suppression of mRNA expression for the gene encoding the 5-HT_{1B} receptor protein in the AMY in females may assist in partially underlying the basis for high aggressiveness previously reported in these experimental females (Poletto et al., 2010a,b). The suppression of the 5-HT_{1B} receptor expression suggests that the inhibitory effects on aggressive behavior mediated through 5-HT postsynaptic signaling may be inefficiently regulated (Davidson et al., 2000; de Boer and Koolhaas, 2005; Nelson and Trainor, 2007). Low mRNA abundance for the protein encoding the 5-HT_{1A} receptor in the AMY of female pigs has been linked to higher aggressiveness observed during the resident–intruder test (D'Eath et al., 2005). Unfortunately, mRNA expression for 5-HT_{1A} receptor was not evaluated in the current study because its genetic code has not yet been sequenced for *Sus scrofa*.

In a study conducted in dogs, the 5-HT_{2A} receptor binding index was higher in the frontal, temporal and cortical brain areas of aggressive/impulsive dogs (Peremans et al., 2003). The up-regulation of 5-HT_{2A} gene expression was only detected in the RN of females, which were also tested as more aggressive (Poletto et al., 2010a,b), suggesting its role in "excitatory" aggression mechanism. Systemic administration of 5-HT_{2A} agonists inhibits 5-HT cell firing in the raphe nuclei of rats (Boothman et al., 2003), and provision of 5-HT_{2A} antagonists has been shown to be very effective in reducing aggression in various human patient populations and animals species (Miczek et al., 2002). We have previously found that 5-HT concentration in the RN was compromised in the dominant female pigs (Poletto et al., 2010b), which may be further aggravating the deficiency in serotonergic signaling in the brain of female pigs.

Similar to the role played by the 5-HT_{2A} subtype, the 5-HT_{2B} receptors also modulate 5-HT release by indirectly stimulating the 5-HT_1 receptor complex, as demonstrated by acute pharmacological inhibition or genetic ablation of the receptor expression (Doly et al., 2008). The serotonergic receptor 5-HT_{2B} has been shown to act presynaptically in the modulation of 5-HT release into the raphe neuron terminal (Doly et al., 2008), thus likely playing an inhibitory role on aggression. The serotonergic 5-HT_{2B} receptors are also involved with the tonic inhibitory control exerted on the mesolimbic and nigrostriatal dopaminergic pathway (Di Giovanni et al., 1999). Both dominant and subordinate pigs had a suppression of the 5-HT_{2B} gene in the midbrain raphe, but the down-regulation was more intense in dominant individuals. A suppression of this receptor expression and thus decreased inhibition of the dopaminergic system, especially in dominant pigs, may be linked with aggressiveness often observed individuals with a higher social status (Jensen, 1982; Poletto et al., 2010b). Furthermore, this same pattern of down-regulation of the 5-HT_{2B} gene expression was detected in the FC and HYP of males and females. However, while the suppression of the 5-HT_{2B} receptor mRNA was greater in the FC of males, its down-regulation was even more evident in the HYP of female pigs. The FC provides inhibitory inputs to neuronal circuitries in the HYP and AMY that might promote aggression (Davidson et al., 2000), and 5-HT release has been shown to decrease in the FC during and after fights (van Erp and Miczek, 2000). It is possible that changes in 5-HT_{2B} expression, or of any other genes, can be accounted for by interference of unidentified variables unrelated to the aggressive profile. Nevertheless, it is important to highlight the potential for a link between aggression and the changes in gene expression observed in the current study, especially in relation to sex differences in the serotonergic system.

The specific role of brain dopaminergic systems in the control of aggressive behavior is contradicting and less understood than the serotonergic system (Miczek et al., 2002; de Almeida et al., 2005b). An intact dopaminergic activity in the mesocorticolimbic system is required, but not exclusively, for aggressive behavior to be initiated and executed, and increases in extracellular dopamine may reflect the motivational aspects of aggression (Miczek et al., 2002; Nelson and Trainor, 2007). In the current study, the abundance of mRNA for the dopamine D_1 receptor varied abruptly between the FC and the RN. In the raphe, females had a two and a half times greater up-regulation of the D1 expression than males. The median and dorsal raphe nuclei neurons are involved in the integration of primary reinforcement and exert tonic inhibition over dopamine-dependent reward circuitry (Rompré and Boye, 1989). In the FC, although both dominant CTL and RAC-fed pigs had a remarkable suppression of the dopamine D_1 receptor mRNA, this down-regulation was less severe in RAC-fed pigs. This finding partially correlates with our previous findings that pigs fed RAC are more aggressive during resident–intruder tests; however, there is no evidence for changes in D1 expression as a function of sex in the FC. Reduced expression of dopamine D1 and D2 receptors in the striatum has been associated with aggression in dopamine transporter knockout mice (Rodriguiz et al., 2004), and D_2 receptors play a key role in controlling of aggression evoked by agonistic stimulation (Nikulina and Kapralova, 1992; Miczek et al., 2002). We could not investigate changes in mRNA expression for D2 receptors in the present study because its genetic codes have not yet been sequenced for pigs. Meanwhile, pharmacological results using agonistic and antagonistic activation of dopamine D_1 receptor have yielded some controversy in relation to the role of this dopaminergic receptor on aggression (Miczek et al., 2002). Antagonists of both the D_1 and D_2 receptors have reduced aggression in male mice (de Almeida et al., 2005b) and male mice only with no previous fight experience decreased aggression upon administration of dopamine D_1 antagonist (Bondar and Kudryavtseva, 2005).

The enzyme MAO-A catalyses the high-affinity oxidative deamination of 5-HT and dopamine into their metabolites, and impinges on aggression by altering neurotransmission rate (Wells and Bjorksten, 1989; Shih et al., 1999). The use of MAO inhibitors decreases the enzyme's activity and consequently increases 5-HT release at the synapse (Wells and Bjorksten, 1989). This inhibitory scenario is somewhat similar to the results found in females, which had a trend for a more intense suppression of the mRNA abundance that encodes for the enzyme MAO-A in the AMY. While this may be a transitory change, it cannot be determined based on the current one time-point study. But previous studies have demonstrated that permanent or chronic MAO-A deficiency appears to cause increased aggressiveness in mice (Cases et al., 1995) and humans (Alia-Klein et al., 2008). Although no changes in MAO-A mRNA abundance were detected between dominant and subordinate pigs in the raphe, it has been shown that mice exposed to recurrent social defeats have an increase of the mRNA level of MAO-A gene in the RN (Kudriavtseva et al., 2004). Nevertheless, while females, especially the subordinate ones, had a down-regulation of MAO-A in the HYP, subordinate males showed an up-regulation in the mRNA abundance for this enzyme in the same brain area. The MAO-A knockout mice have been shown to exhibit increased aggression despite the elevated availability of 5-HT in the brain (Cases et al., 1995).

In summary, females showed more suppression of the 5-HT receptor genes and MAO-A gene in the brain areas tested than males; the mRNA levels for 5-HT_{1B} were only suppressed in the AMY of females, and 5-HT_{2B} was also down-regulated in the RN, FC and HYP of females and in the RN of dominant pigs; furthermore, MAO-A mRNA expression was suppressed in the RN, AMY and HYP of females. On the other hand, 5-HT_{2A} expression only changed in the RN and was more up-regulated in females than males. Meanwhile, the D_1 receptor gene expression varied in the RN and FC as a function of RAC dietary treatment and its interactions with sex and social rank of the pigs. This variation in expression of genes involved in regulation of the serotonergic and dopaminergic systems detected mainly in female pigs, which were also found more aggressive, support the role of these underlying molecular mechanisms in aggression regulation.

4. Experimental procedures

4.1. *Animal housing and experimental design*

A total of 64 domestic pigs (*Sus scrofa*), including 32 male pigs (known as barrows) and 32 female pigs (known as gilts), at approximately 6 months of age, were used for this study. Pigs naturally reach puberty between 5 and 7 months of age, and at around this time are also sent to slaughter for meat production. The experimental pigs were selected from a population of 170 animals according to similarity in initial body weights and in a manner to avoid parental relatedness, and were assigned to 1 of 4 blocks accordingly. There was a 2-week interval between the initiation of blocks 1 and 2 and blocks 3 and 4 on the trial. Each block was balanced for sex, dietary treatment, and body weight, and was allocated into 1 of 2 rows of 8 adjacent pens, which housed 4 unrelated pigs in each pen. Both rows of pens were located in the same room of the Purdue University Swine Evaluation Unit and were separated by a 1.5 m-wide aisle. Pens in each row were organized so they alternated sex and dietary treatment order within a body weight block to remove any potential location effects within the building. Each pen was 1.8 m × 3.0 m with the rear two-thirds of the floor being fully-slatted concrete and the front one-third of the floor being plastic-coated expanded metal. A single nipple drinker was mounted in the middle of one of the pen sides, and a single-spaced feeder was situated at the front of each pen. The pigs had water and feed provided *ad libitum*. The room was ventilated naturally and temperature maintained at a minimum of 18.5 °C as the trial was carried out during the winter time (January to March). The experimental procedures used in this study were approved by the Purdue University Animal Care and Use Committee and animals were housed in accordance with FASS (1999) guidelines.

Pigs were assigned to their pens at day −14, when mixing of unfamiliar pigs in each pen was carried out. Within each

block, pigs in each pen were assigned to the dietary treatments that were either control (CTL) or RAC feeding. Pigs on both dietary treatments were initially fed the same standard basal diet for 2 weeks (from day –14 to 0). Thereafter, CTL pigs continued to receive the standard basal diet, whereas RAC-fed pigs had part of the starch fraction of the basal diet substituted by ractopamine hydrochloride (Paylean®, Elanco Animal Health, Greenfield, IN). Ractopamine was delivered using a "step-up" feeding program where RAC-fed pigs received the compound added to their diet at 5 mg/kg (5 ppm) for 2 weeks (day 0 to 14), and then it was increased to 10 mg/kg (10 ppm) for the final 2 weeks preceding slaughter (day 14 to 28). The basal diet was corn–soybean meal based (17.6% Crude Protein, 1.1% Lysine) and fed in meal form and provided *as-fed* basis. A detailed table with the composition of the experimental basal diet provided to the finishing pigs is available in Poletto et al. (2009). Tryptophan was provided in the diet for all pigs at a 0.2% inclusion rate (Schinckel et al., 2003). We have previously measured concentrations of 5-HT and tryptophan in the blood (Poletto et al., 2010a) and found that female pigs tended to have lower peripheral 5-HT concentrations than male pigs, whereas tryptophan levels were not different. Similarly, concentrations of 5-HT in all four brain areas in which changes in gene expression were examined (Poletto et al., 2010b) for all 32 experimental pigs, with the only differences being lower 5-HT concentrations in the frontal cortex of female pigs compared to males ($P<0.05$), and lower 5-HT in the raphe nucleus of dominant females compared to subordinate females ($P<0.05$).

4.2. *Assignment of social rank*

Upon pen assignment at mixing, pigs within each pen were individually marked for identification during behavioral observation. Behavior recording of all pigs in each pen started at the time of mixing, at 2 weeks prior to the start of the dietary treatment, and continued for 36 h, using ceiling-mounted cameras (Panasonic WV-CD110AE, Matsushita Electric Industrial Co. Ltd., Osaka, Japan) attached to a digital video recording system (IPD-DVR816, Inter-Pacific, Inc., Northbrook, IL). No software was used to extract the behavioral data; an observer, the same person, watched all recorded videos. This behavioral information was used to investigate dominance hierarchy formation, i.e., the social rank of pigs within their new home pens.

The top dominant and the bottom subordinate pigs ($n=2$) in each pen of 4 pigs were determined using continuous focal sampling of all pigs for the 36 h period, concentrating on the outcome of all agonistic encounters/interactions taking place within each pen. An agonistic interaction was defined as an event in which one pig directed offensive "forced" behaviors against another pig in the group, during which the identification of initiators and receivers of the first attack were recorded. Offensive (i.e., bites, head knocks, pursuits, and threats) and defensive behaviors (i.e. freeze, avoid, and flight) displayed and the duration of each agonistic social interaction were recorded. A threat was defined as a pig, with or without its mouth open, displaying a vigorous lunging movement of its head towards another pig without making physical contact. The loser of each agonistic interaction was deemed when a pig clearly showed any of the defensive behaviors mentioned above that led to termination of the social interaction.

The following criteria were used to determine the social rank of pigs within each pen, a) dominance matrix: estimated by the number of occasions when a pig was supplanted versus the number of occasions that that pig supplanted others, using the outcomes of all pair-wise interactions (Martin and Bateson, 1993), and b) level and success of interaction: measured by counting the total frequency of agonistic interactions that each pig was involved in and the percentages of interactions which that pig won or lost, as evidenced by supplanting or resisting the displacement by the opponent (Bradshaw et al., 2000). Outcomes from both approaches, but more robustly the level and success of interaction, assisted in determining the top dominant (highest scores, >0.66) and bottom subordinate (lowest scores, <0.33) pig in each pen. The remaining two pigs in the pen, with middle scores, were categorized as intermediate in social rank. Monitoring of social rank was carried out weekly during the study by behavioral assessment of agonistic interactions in the home pen and their outcomes, and information is published elsewhere (Poletto et al., 2010a).

4.3. *Tissue preparation and RNA extraction*

On day 31, three days after the end of the trial (day 28), the 32 pigs (16 males and 16 females) classified as the top dominant and the bottom subordinate from each pen were transported for approximately 16 km to the slaughter facility at the Purdue University, West Lafayette campus, for brain sample collection. Between day 28 and 31, RAC-fed pigs continued to be fed *ad libitum* with the 10 mg/kg RAC-added diet, while CTL pigs were maintained on the same basal diet as given during the 4-week trial. Since blocks 1 and 2 went on trial simultaneously but 2 weeks apart from blocks 3 and 4, 8 dominant and 8 subordinate pigs were slaughtered each time. At the day of slaughter, 8 pigs (from one block – 1 or 3) were transported to the slaughter plant at 0700, while the other 8 pigs (from block – 2 or 4) were then transported to the slaughter plant at 0900. Gentle handling with a board was used to move pen mate pigs (dominant and subordinate pigs) in pairs from their home pen onto a flatbed truck. Each pair was allocated to a subdivision of the trailer that prevented mixing with the other experimental pigs during transport. Upon arrival at the facility, pen mate pairs were also kept in subdivisions in the holding area to prevent mixing for a maximum of 1 h preceding stunning. Water and feed were not available during lairage although pigs were wetted with a hose.

At slaughter, pigs were stunned and exsanguinated one at a time. Each skull was then opened with an electrical saw and brains dissected within a 10 min interval *post-mortem*. The brain areas of interest AMY, FC (collected from the superior frontal sulcus region), HYP and RN were dissected from the right hemisphere based on the landmarks of a pig brain atlas (Felix et al., 1999). Following dissection, the samples were placed in RNA*later*™ (Ambion, Austin, TX) to protect RNA integrity, and then immediately frozen in dry ice and stored at –80 °C until processing. At the time of processing, the RNA*later*™ was removed from the samples according to manufacturer's guidelines. Total RNA from individual brain

area samples was isolated using the Ribo Pure Kit (Ambion) following the manufacturer's instructions and all samples were subjected to DNAse treatment (Turbo™ DNAse, Ambion). Quantity and purity of isolated RNA samples were analyzed using a spectrophotometer (GeneQuant, Biochrom Ltd., Cambridge, UK).

4.4. *Quantitative real-time RT-PCR*

Quantitative real-time reverse transcriptase polymerase chain reaction (Q-RT-PCR) was performed to examine the relative abundance of mRNA encoding for the proteins of the serotonin receptors 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, the dopamine receptor D_1 and the enzyme MAO-A in the AMY, FC, HYP, and RN of pigs. The Q-RT-PCR was performed as described previously (Poletto et al., 2006a,b; Gibson et al., 1996). A total of 2 μg of total RNA from each sample was reverse transcribed with oligo $(dT)_{18}$ and SuperScript II reverse transcriptase (Invitrogen Life Technologies Corp., Carlsbad, CA). A spectrophotometer (GeneQuant) was used to determine quality and quantity of the resulting complementary DNA. Primer sequences for Q-RT-PCR were designed using Primer Express 2.0 Software (Applied Biosystems, Foster City, CA) and synthesized by Operon Biotechnologies, Inc. (Huntsville, AL). Primer sequences for tested and control genes are shown in Table 2.

A total of 30 ng of template cDNA and 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems) were employed in each real-time reaction. The primer mix (forward and reverse sequences) was used in a concentration of 5 μM. *Sus scrofa* 18S ribosomal RNA was used as the control gene for the FC, RN and HYP, while beta actin was selected as the control gene for the AMY, and both endogenous genes used for normalization purposes. In order to select these housekeeping genes, samples from all four brain areas belonging to pigs assigned to both dietary treatments, sexes, and social ranks (2×2×2 combination) were tested against both beta-actin and 18S. The mean Ct between duplicate runs for every testing sample was averaged, and the mean standard deviation among all Cts was calculated. The control gene with the smallest Ct standard deviation was chosen. For instance, the standard deviation of mean Cts for amygdala samples was 0.5644, and the standard deviation among all Cts for 18S was 0.8079.

All Q-RT-PCR reactions were performed in triplicate using a template from individual animals and from each brain area in each reaction. Standard deviation among the triplicates was smaller than 0.30. A relative standard curve was used as the Q-RT-PCR quantification method (Livak, 1997). The standard curve was constructed using the following amounts of cDNA (in duplicate): 320, 160, 80, 40, 20 and 10 ng. A single control sample from each brain area was chosen to be used as the template for the standard curves (Fig. 1). Standard curves for the control genes and genes of interest were incorporated into every run and the R^2 for all standard curves was greater than 0.99. The Q-RT-PCR reactions were performed and analyzed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

4.5. *Q-RT-PCR data analysis*

The relative standard curve quantification software yielded 3 expression values for the tested and reference genes in each sample. These three technical replicates were averaged and the result for each tested gene was divided by the corresponding value of the control to obtain the normalized gene expression in each biological sample. For further analysis, the log-transformation of the normalized quantity for each sample was considered as the response variable. The relative expression of the 5 genes was analyzed simultaneously using multivariate linear mixed model (Littell et al., 1996) using SAS software (SAS Institute, Inc., Cary, NC). Dietary treatment (RAC and CTL), sex (male and female), social rank (dominant and subordinate), gene (5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, D_1 and MAO-A) and all possible interactions were set as fixed effects. Pig and its interactions were considered as a random effect. All the variances were estimated and the model assumptions (e.g. normality) were assessed. The main or simple effect contrasts were computed depending on the significance of the higher order interactions, and statistically significant interactions are shown in the figures. Fold-changes (ratios) were obtained by back transforming the linear estimates. The back transformation of the SEM was obtained by taking the anti-log of the mean plus or minus the standard error of the mean (SEM). For presentation of the results in which the gene expression was lower in the first dietary treatment, sex, or social rank effect compared to the second, the ratio (<1) was divided by −1 (−1/ratio) to yield a negative relative expression value. Positive fold-changes in gene expression are represented by positive values while negative fold-changes are shown as negative values. Differences in

Table 2 – Primer sequences used for Q-RT-PCR to measure gene expression in the amygdala, frontal cortex, hypothalamus and raphe nuclei of pigs.

Gene name *	GenBank accession no.	Forward primer (5′→3′ end)	Reverse primer (5′→3′ end)
5-HT_{1B}	AF188626	TCGGACATCACCTGTTGCA	AGCGGTCCAAAGCGATGA
5-HT_{2A}	S78208	GCAAGGTGCTGGGCATAGTC	ACGGCCATGATGTTGGTGAT
5-HT_{2B}	Z48174	GCCCTGCCTGGTTATTTCTTG	CCACTGAAATGGCACAAAGATG
D_1	NM_001123108	GCAGCTCAGCTGGCACAAG	CTTGCCCAGGGAAGTGACA
MAO-A	AY563632	CAATGGAGCGGTTACATGGA	TGACACCTTCCCCAAAGCA
18S	AY265350	TCCGGAATCGAACCCTGAT	GTAGTCGCCGTGCCTACCA
Beta actin	AF054837	CTCCTTCCTGGGCATGGA	CGCACTTCATGATCGAGTTGA

* 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2B} are classified as serotonergic receptors; D_1 is classified as a dopaminergic receptor type 1; MAO-A is defined as a monoamine oxidase type A.

fold-change with a $P<0.05$ were considered statistically significant, while $P<0.10$ were considered a trend towards statistical significance. Fold-change values are presented in figures as the mean differences with the corresponding SEM.

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